Food Research International 98 (2017) 20–33

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Targeted and untargeted high resolution mass approach for a putative profiling of glycosylated simple phenols in hybrid grapes

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ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form 25 November 2016
Accepted 12 January 2017
Available online 13 January 2017

Keywords:
LC-HRMS
Untargeted
SPE
Glycosylphenol
European grapes

ABSTRACT

Vitis vinifera is one of the most widespread grapevines around the world representing the raw material for high quality wine production. The availability of more resistant interspecific hybrid vine varieties, developed from crosses between *Vitis vinifera* and other *Vitis* species, has generated much interest, also due to the low environmental effect of production. However, hybrid grape wine composition and varietal differences between interspecific hybrids have not been well defined, particularly for the simple phenols profile. The dynamic of these phenols in wines, where the glycosylated forms can be transformed into the free ones during winemaking, also raises an increasing health interest by their role as antoxidants in wine consumers.

In this work an on-line SPE clean-up device, to reduce matrix interference, was combined with ultra-high liquid chromatography-high resolution mass spectrometry in order to increase understanding of the phenolic composition of hybrid grape varieties. Specifically, the phenolic composition of 4 hybrid grape varieties (red, Cabernet Cantor and Prior; white, Muscaris and Solaris) and 2 European grape varieties (red, Merlot; white, Chardonnay) was investigated, focusing on free and glycosidically bound simple phenols and considering compound distribution in pulp, skin, seeds and wine. Using a targeted approach 53 free simple phenols and 7 glycosidic precursors were quantified with quantification limits ranging from 0.001 to 2 mg Kg^{-1} and calibration R^2 of 0.99 for over 86% of compounds. The untargeted approach made it possible to tentatively identify 79 glycosylated precursors of selected free simple phenols in the form of -hexoside (N = 30), -pentoside (21), -hexoside-hexoside (17), -hexoside-pentoside (4), -pentoside-hexoside (5) and -pentoside-pentoside (2) derivatives on the basis of accurate mass, isotopic pattern and MS/MS fragmentation.

1. Introduction

The European grapevine belongs to the *Vitis vinifera* botanical species which is the most widely used around the world for wine production (about 7.4 million ha; FAO, 2002). Unfortunately, *Vitis vinifera* grapes are susceptible to various diseases, mildew (Reisch, Owens, & Cousins, 2012) and insects' damage, Phylloxera in particular, and despite being grafted onto resistant rootstocks, they need frequent treatments against several pathogens. However, massive use of pesticides is unsustainable from an environmental and economic point of view, as well as leading to pesticide resistance.

An environmentally friendly solution to the problems of pesticide pollution could be represented by hybrid grape varieties crossing *Vitis vinifera* and other *Vitis* spp. (e.g. *V. riparia*, *V. labrusca*, *V. rupestris*; Sun, Gates, Lavin, Acree, & Sacks, 2011), which are selected to obtain higher tolerance or resistance to abiotic stress, e.g. temperature (Burns et al., 2002), and biotic stress (Slegers, Angers, Ouellet, Truchon, & Pedneault, 2015). Due to their low sugar and tannin content (Harbertson et al., 2008; Springer & Gavin, 2014), foxy taste and several off-flavours (Rapp, 1990; Sun et al., 2011), only a reduced number of hybrid varieties are authorised in European countries for the production of wines not included in Protected Designations of Origin (European Community Regulation, 2008; D.Lgs. 61/2010). Despite their potential interest as a result of the low environmental impact, the phenolic profile of hybrid grape varieties has not been extensively evaluated nor have varietal differences between interspecific hybrids been well defined.

The effects on health attributed to wine consumption are principally due to its high content in terms of phenols, which have been demonstrated to have anti-inflammatory, anti-oxidant and cardioprotective effects (Middleton, Kandaswami, & Theoharides, 2000). Furthermore, phenolic compounds, widely accumulated in the skin and seeds (Poudel, Tamura, Kataoka, & Mochioka, 2008), contribute towards defining wine taste (Arnold & Noble, 1978), together with volatile

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http://dx.doi.org/10.1016/j.foodres.2017.01.011
0963-9969/

compounds produced during fermentation and those transferred from wood during ageing (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006, chap. 7). Structurally, they range from low-molecular weight simple phenols to more complex compounds, and many of them can be found in the form of mono- and disaccharides or as ester and methoxy derivatives (Shahidi & Naczk, 1995). Free phenols can directly affect wine astringency or bitterness, and can be involved in the redox equilibrium of wine (Keller, 2009), while flavourless glycoconjugates are considered aroma precursors, since they can be hydrolysed during winemaking or ageing, releasing the corresponding volatile aglycones (Williams, Strauss, Wilson, & Massy-Westropp, 1982).

Spectrophotometric and chromatographic methods have been developed for phenol analysis (Naczk & Shahidi, 2004), and while the former are rapid but less specific assays, chromatographic approaches are able to identify and quantify single phenols (Larcher et al., 2007; Barnaba et al., 2015). Gas chromatographic methods require some phenolic compounds to be transformed into more volatile derivatives, while high-performance liquid chromatographic approaches do not require a derivatisation step, and are thus less time-consuming. In the case of phenolic glycoconjugates, analyses generally involve complex extraction and hydrolysis procedures aimed at isolating aglycones for subsequent direct analysis (Williams et al., 1982). Recent works (Perestrelo et al., 2012; Di Lecce et al., 2014; Barnaba et al., 2016) have tried to structurally define grape phenolic glycoconjugates through liquid chromatography coupled with tandem mass spectrometry.

The study aims to tentatively define the free and glycosidically simple phenolic composition of 4 hybrid grapes, describing their distribution in skin, pulp and seed in comparison to 2 European varieties, using a targeted and untargeted UHPLC-high resolution tandem mass approach. Wines from hybrid varieties were also investigated in order to define the specific oenological composition of these products.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN; LC-MS grade, 99.9%), methanol (LC-MS grade, 99.9%), formic acid (MS grade, 98%) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol, 99.5%) were supplied by Fluka (St. Louis, MO, USA). Acetic acid (99%), L-glutathione reduced (99%), *p*-nitrophenol (99%), D-(+)-gluconic acid-δ-lactone (99.0%) and sodium azide (99.5%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The targeted phenolic compound suppliers are summarised in Table 1. Deionized water was produced using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Eleven standard stock solutions (200 mg L^{-1} of each phenol) were prepared in water-methanol, with organic solvent ranging from 15 to 55% according to the component's solubility. L-glutathione reduced and DL-dithiothreitol were added as antioxidant agents (2.5 g L^{-1} each). Merging an aliquot from each stock solution, a more diluted solution (10 mg L^{-1} for each phenol) was obtained and used to prepare calibration solutions in the range 0.0001–10 mg L^{-1} (Barnaba et al., 2015). All solutions were stored at −4 °C.

Instrument mass calibration was performed using a standard mixture composed of sodium dodecyl sulphate, sodium taurocholate (2.6 mg L^{-1} and 4.9 mg L^{-1} respectively; Pierce® ESI Negative Ion Calibration Solution, Rockford, IL, USA), formic and acetic acids (5 mg L^{-1} each).

2.2. Samples and sample extraction

This study considered 2 white (Solaris and Muscaris) and 2 red (Cabernet Cantor and Prior) hybrid grape varieties, 2 European ones (Chardonnay and Merlot), and wines from selected hybrid grapes. The Solaris variety, originally known as Fr. 240-75, is a cross between Merzling (mother) and Gm 6493 (Zarya severa x Muskat Ottonel; father), while Muscaris, originally known as Fr. 493-87, is a cross between Solaris (mother) and Muskateller (father). Cabernet Cantor, originally known as Fr. 523-89r, derives from crossing of Seibel 70-53 (mother) with Solaris (father), while Prior, originally known as Fr. 484-87r and then as Fr. 455-83r, derives from the crossing of Bronner (Merzling x Gm 6494; father) and the product of a cross between Joannès-Seyve 23-416 and Pinot Noir (mother) (http://www.wine-searcher.com/grape-varieties.lml).

Solaris, Muscaris and Cabernet Cantor grapes were sampled in triplicate from 2 experimental plots, located in Rovereto (TN, Italy; latitude: 45° 52′ 33.96″, longitude: 11° 1′ 4.12″, altitude: 204 m, AMSL; trellis system, 3 × 0.9 m) and Telve (TN, Italy; latitude: 46° 3′ 42.08″, longitude: 11° 28′ 41.59″, altitude: 548 m, AMSL; guyot, 2 × 0.8 m), respectively. Samples in triplicate were also collected from Telve for Prior, and from Rovereto for Chardonnay and Merlot.

The grape samples were picked at technological ripeness (Muscaris grapes were harvested in the Rovereto plot on 3 September 2015, with sugar content = 23.3°Brix, pH = 3.36, total acidity = 4.8 g tartaric acid L^{-1}, and in the Telve plot on 10 September 2015, with values of 24.8, 3.32 and 4.3 respectively; Solaris: harvested in the Rovereto plot on 28 August 2015 with values of 24.7, 3.30 and 5.2, and in the Telve plot on 31 August 2015 with values of 23.1, 3.30 and 5.8 respectively; Cabernet Cantor: harvested in the Rovereto plot on 22 September 2015 with values of 21.5, 3.40 and 4.7, and in the Telve plot on 16 September 2015 with values of 23.6, 3.26 and 6.3 respectively; Prior: harvested in the Telve plot on 16 September 2015 with values of 20.4, 3.28 and 7.2 respectively; Chardonnay: harvested in the Rovereto plot on 9 September 2015 with values of 21.1, 3.29 and 5.0 respectively; Merlot: harvested in the plot of Rovereto on 10 September 2015 with values of 22.3, 3.40 and 4.6 respectively).

In order to prepare the separate pulp, skin and seed fractions, roughly 600 sound and intact berries from each grape sample (3 *per* plot) were randomly collected from the top, sides, middle and bottom of 15 ripe bunches, and stored at −20 °C until fractions preparation. Before analysis, 25 berries were randomly collected from each one of the 600-berry samples and weighed. Then the frozen berries were separated into skin, pulp and seed fractions using pincers. Two grams of each fraction were extracted with 20 mL of the extraction solvent, dispersing the suspension with Ultra Turrax (24,000 rpm, 30 s; T 25 basic, IKA®-Werke GmbH & Co. KG) and shaken overnight (Rotoshake; C. Gerhardt GmbH & Co. KG). The extraction solvent was a solution of H_2O-MeOH 1:1, added with NaN_3 (0.01%, p/v) as antifermentative agent, L-glutathione reduced and DL-dithiothreitol (0.25% each, p/v) as antioxidant agents, and D-(+)-gluconic acid-δ-lactone (0.50%, p/v) in order to inhibit β-glucosidase (Günata, Biron, Sapis, & Bayonove, 1989). After centrifugation (4000 rpm, 45 min; IEC CL31 Multispeed, Thermo Fisher Scientific), the supernatant was filtered with 0.45 μm PTFE filter cartridges (Sartorius AG, Goettingen, Germany), diluted 2 times with water and added of the internal standard (*p*-nitrophenol, 500 μg Kg^{-1}). Only seed extracts were analysed at two dilution levels (2 and 300 times), in order to cover the very different concentration ranges of the 60 compounds (from a few μg/Kg of aesculetin, methyl vanillate or syringic acid to several mg/Kg of epicatechin, catechin or gallic acid; Di Lecce et al., 2014).

The wines (Solaris, N = 2; Muscaris, 2; Cantor, 2; Prior, 1; 2015 harvest) were produced at the experimental winery of the Mach Foundation from 40 kg of ripe and sound hybrids grapes. After destemming, white varieties were pressed and cold settled for 24 h. The racked must fermented then at 20 °C with EC1118 yeast strain (200 mg L^{-1}; Lallemand, Verona, Italy). At the depletion of sugar content (<1 g L^{-1}), wines were kept at 4 °C until analysis. Red varieties were destemmed and inoculated keeping the same strain and doses as white wines. After 7 days of skin contact maceration, marcs were removed and the resulting wines once the alcoholic fermentation was ascertained, were inoculated with PN4 selected lactic bacteria